STIC-ILL

NPL

From: Canella, Karen
Sent: Thursday, February 12, 2004 4:18 PM
To: STIC-ILL
Subject: ill order PCT/US03/24585

Art Unit 1642 Location Remsen 3A29 (office); 3C18 (mailbox)

Telephone Number 272-0828

Application Number PCT/US03/24585

1. Journal of Cell Science, 2000 Oct, 113, Pt 19, pp. 3365-3374

2. Nature Medicine:
2001 Mar, 7(3):297-303
1998 May, 4(5):594-600
1996 Jan, 2(1):52-58

3. Advances in Experimental Medicine and Biology, 2001, Vol. 495 (progress in basic and clinical immunology), pp. 349-354.

4. Europen Journal of Immunology, 1998, 28(5):1636-1644

5. Cancer Biotherapy & Radiopharmaceuticals, 2000 Apr, 15(2):185-194

6. CAPLUS COPYRIGHT 2004 ACS on STN
ACCESSION NUMBER: 1999:16570 CAPLUS
DOCUMENT NUMBER: 130:236031
TITLE: Dendritic cell-derived exosomes: potent immunogenic cell-free vaccines
AUTHOR(S): Zitvogel, Laurence; Regnault, Armelle; Lozier, Anne; Raposo, Graca; Amigorena, Sebastian
CORPORATE SOURCE: Laboratoire d'Immunologie Cellulaire, Departement de Biologie Clinique, Institut Gustave Roussy, Villejuif, Fr.
SOURCE: Dendritic Cells (1999), 643-652. Editor(s): Lotze, Michael T.; Thomson, Angus W. Academic: San Diego, Calif.
CODEN: 67DCAA
DOCUMENT TYPE: Conference; General Review
LANGUAGE: English

7. Hematology and Cell Therapy, 1998 Apr, 40(2):87-89

Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming

JOSEPH WOLFERS[1], ANNE LOZIER[1], GRAÇA RAPOSO[3], ARMELLE REGNAULT[2], CLOTILDE THÉRY[2], CAROLE MASURIER[1], CAROLINE FLAMENT[1], STÉPHANIE POUZIEUX[4], FLORENCE FAURE[2], THOMAS TURSZ[1], ERIC ANGEVIN[1], SEBASTIAN AMIGORENA[2] & LAURENCE ZITVOGEL[1]

[1]*Immunology Unit, Department of Clinical Biology, Institut Gustave Roussy, Villejuif, France*
[2]*Unité INSERM U520 and* [3]*UMR 144 CNRS Institut Curie, Paris, France*
[4]*APCells SA, Institut Gustave Roussy, Villejuif, France*
J.W. & A.L., contributed equally to this study.
Correspondence should be addressed to S.A. or L.Z.; email: sebastian.amigoreana@curie.fr; zitvogel@igr.fr

The initiation of T-cell–mediated antitumor immune responses requires the uptake and processing of tumor antigens by dendritic cells and their presentation on MHC-I molecules. Here we show in a human *in vitro* model system that exosomes, a population of small membrane vesicles secreted by living tumor cells, contain and transfer tumor antigens to dendritic cells. After mouse tumor exosome uptake, dendritic cells induce potent CD8⁺ T-cell–dependent antitumor effects on syngeneic and allogeneic established mouse tumors. Therefore, exosomes represent a novel source of tumor-rejection antigens for T-cell cross priming, relevant for immunointerventions.

Tumor cells express an array of antigens recognized by CTLs (cytotoxic T lymphocytes)[1,2]. The nature of the tumor antigens that actually mediate efficient immune responses leading to tumor rejection remains unclear[3]. Tumor transplantation studies in mice using carcinogen-induced neoplasia demonstrate that antigens resulting from mutations incidental to the oncogenic process are responsible for protective immunity in tumor bearing animals[4–6]. Shared tumor antigens in murine studies have not been well-described[7], and although they can serve as tumor-rejection antigens, cross-protection between such antigen-positive tumor cells has not been observed[8].

In contrast, most human tumor antigens reported to date are nonmutated, shared or tissue-restricted antigens[1,2,9]. This indicates that cross-protection among different tumors might be possible[10] and use of a set of shared tumor antigens may allow vaccination against a broad variety of tumor types.

Most of the current reports pertaining to a source of relevant tumor antigens examine antigens derived from dying tumor cells[11–18]. Nevertheless, the proof of principle of shared tumor rejection antigens promoting cross-protection among various tumor models has not been made in such settings. The relevant pathways for efficient tumor-antigen transfer from tumor cells to dendritic cells remain controversial.

We and others have previously reported that B lymphocytes[19] and dendritic cells[20] secrete membrane vesicles of endosomal origin called 'exosomes' upon fusion of multivesicular bodies (MVB) with the plasma membrane. Such exosomes display a discrete set of proteins involved in antigen presentation, that is, major histocompatibility complex class I and II (MHC-I and MHC-II), costimulatory and tetraspan molecules (CD63, CD82) and are selectively enriched in molecules potentially involved in effector cell targeting, that is, CD11b, lactadherin and CD9 molecules[21,22]. Peptide-pulsed dendritic-cell–derived exosomes elicit potent antitumor immune responses in tumor-bearing mice[20].

Here we show that both human and mouse tumor cells constitutively release 60–90-nm membrane vesicles, similar to dendritic-cell–derived exosomes in their morphology, density and expression of certain membrane markers. When loaded onto human dendritic cells, tumor-derived exosomes transfer shared tumor antigens allowing specific human CTL activation *in vitro* and CD8⁺ T-cell–dependent cross-protection among different, poorly immunogenic mouse tumors *in vivo*.

Tumor cell lines secrete exosomes

We assessed whether tumor cells, like B lymphocytes[19] and dendritic cells[20], harbored MVB which would potentially fuse with the cell surface and prompt release of the internal vesicles. Electron microscopy analysis of the murine mammary adenocarcinoma cell line TS/A (H-2^d) (Fig. 1*a*) and of P815 mastocytoma cells (H-2^d) (data not shown) revealed numerous MVB. These compartments contained internal vesicles and were strongly labeled with an anti-lysosome associated membrane protein (LAMP) monoclonal antibody (Fig. 1*a*). Differential ultracentrifugation of TS/A or P815 culture supernatants yielded pellets consisting of a similar population of vesicles 60–90-nm in diameter (Fig. 1*b* and *c*) resembling APC (antigen-presenting cell)-derived, cup-shaped exosomes. The yield of exosome production by tumor cells was lower than in dendritic cells (0.3–0.5 μg versus 1–2 μg per million cells in 48 h).

Tumor-derived exosomes bear MHC-I molecules and LAMP1 as detected by western blotting, but lacked the endoplasmic reticulum (ER) resident protein calnexin (data not shown). The vesicles floated at a density of 1.14 g/ml in continuous sucrose gradient (data not shown), as previously reported for APC-derived exosomes[19,21]. Metabolic labeling experiments using P815 and TS/A tumor cells revealed that exosomes display a discrete set of proteins compared with total cell membranes (Fig. 1*d*). Exosomes were also found to be constitutively secreted by other tumor cell lines such as colon carcinoma MC38 (H-2^b), leukemia L1210 (H-2^d), mesothelioma AK7 (H-2^b) and various human pri-

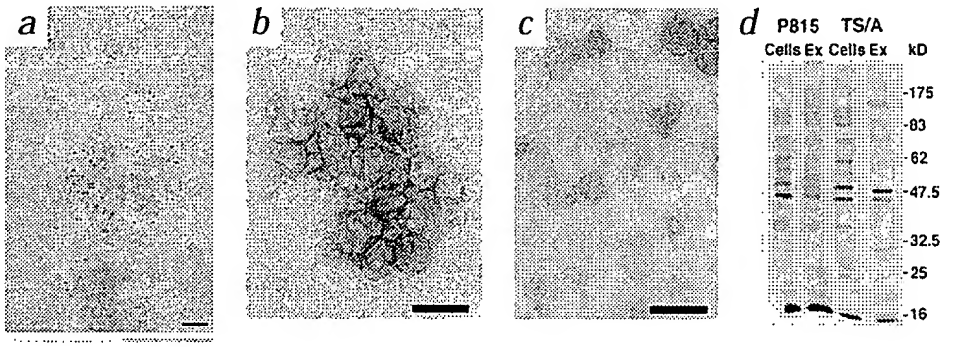

Fig. 1 Tumor cells display multivesicular bodies and secrete exosomes. *a*, Immunoelectron microscopy of TS/A cells display multivesicular endosomes. *b* and *c*, Electron microscopy of pellets obtained after differential ultracentrifugation of the cell culture supernatants of tumor cells. *b*, TS/A cell pellets consist of aggregates of membrane vesicles homogeneous in size (approximately 80 nm). *c*, P815 cell pellets are composed of membrane vesicles of 40 to 100nm. Bars = 100nm. *d*, Overall protein composition of tumor-cell-derived exosomes. Exosomes purified from the supernatant of metabolically labeled TS/A and P815 cells were run on a 10% SDS gel (Ex), together with lysates (cytosolic and membrane components) from whole cells (Cells) in the absence of detergent. The dried gel was autoradiographed for 24 h (proteins above 45 kD) or 48 h (proteins below 45 kD).

mary tumor cultures (data not shown). Induction of apoptosis (by cisplatin or ultraviolet irradiation) decreased the amount of exosomes produced by P815 and TS/A (data not shown). This suggests that exosomes are only produced by living tumor cells. Therefore, established murine tumor cell lines in culture constitutively secrete membrane vesicles morphologically analogous to the exosomes released by hematopoietic cells.

Exosomes are immunogenic across histology and MHC-I barriers

As exosomes derived from tumor peptide-pulsed dendritic cells were found to be immunogenic[20], we tested the immunogenicity of tumor-derived exosomes. In prophylaxis studies, we assessed whether ten times the minimal tumorigenic doses (MTD) of irradiated TS/A cells[32], three days apart, could protect the syngeneic BALB/c mice against lethal challenge using 1×10^5 live TS/A, and we compared such protective antitumor effects with those promoted by purified TS/A-derived exosomes (10–20 μg). Indeed, whereas we found the TS/A tumor model to be immunogenic with up to 60% efficient long-term survival using irradiated tumor cells, autologous exosomes proved even more efficient with 90% tumor-free mice in primary immune responses (Fig. 2*a*). This difference could not be accounted for by different dosages of total tumor-cell-derived proteins injected between whole tumor cells and exosomes, as 1×10^6 irradiated cells contain 50-fold excess of proteins over the 20 μg of exosomal proteins injected. In addition, injection of 20 μg of total tumor-cell proteins (that is, equivalent to 2×10^4 irradiated tumor cells) could not protect the host against a lethal challenge whereas this amount of exosomal proteins could. In accordance with the well-recognized 'specificity' of the immunization mediated by irradiated cells, we found that irradiated irrelevant MC38 (H-2^b) adenocarcinoma could not cross-protect BALB/c mice against a lethal challenge with TS/A breast cancer (H-2^d) (Fig. 2*b*). Surprisingly, MC38-derived exosomes were as efficient as the autologous TS/A-derived exosomes to protect the host against TS/A establishment (Fig. 2*b*).

Cross-protection using exosome-based immunization could not be accounted for by technical artifacts, which was shown by three independent strategies: 1) mouse tumor-cell lines were cultured in serum-free culture medium for 48 hours and cell supernatants were collected for exosome purification; 2) the complete culture medium was pre-ultracentrifuged to reduce serum-related contaminants before tumor-cell culture, and under these conditions of exosome purification, similar antitumor effects were observed in the TS/A-MC38 model (data not shown); 3) 'mock' exosomes prepared from culture medium were used for prophylaxis studies without significant antitumor effects (Fig. 2*a*, 'CM pellet'). Finally, endogenous murine retroviruses were not observed by electron microscopy, and all cells were found to be free of mycoplasma.

Complete and reciprocal cross-therapeutic immunization

It is likely that cross-presentation of exosomal tumor antigens is mediated by host-derived APCs. Therefore, in order to achieve therapeutic activity, we tested the efficacy of *ex vivo*-propagated, bone-marrow-derived dendritic cells (BM-DCs) loaded with tumor-derived exosomes. 5×10^5 syngeneic BM-DCs (H-2^d) loaded with TS/A derived exosomes were injected once or twice into the ipsilateral flank of a day 3-4 established TS/A (H-2^d,

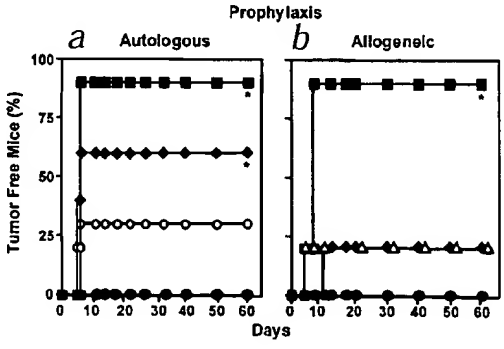

Fig. 2 Cross-immunizations using tumor-derived exosomes. *a*, Autologous tumor-derived exosomes prevent TS/A (H-2^d, breast cancer, BALB/c)-tumor establishment. Mice were inoculated with either saline (●), 1×10^6 γ-irradiated TS/A tumor cells (◆), TS/A tumor-derived exosomes (■) or pellets collected from cell-free culture medium (CM pellet, ○). *b*, Allogeneic tumor-derived exosomes prevent TS/A tumor establishment. Similar experimental setting as *a* using intradermal inoculations of exosomal proteins (■) derived from MC38 cells (H-2^b, C57BL/6, colon cancer) or equivalent protein amounts of MC38 lysates (△) or 1×10^6 γ-irradiated MC38 cells (◆) or saline (●). 10 d after the last immunization (day 0), challenge with twice the MTD of live TS/A was performed. Each graph represents the data pooled from 3 independent experiments. 15 mice/individual group were used for statistical analyses. *, statistical significance at 95% confidence against controls (saline) using Fisher's exact method.

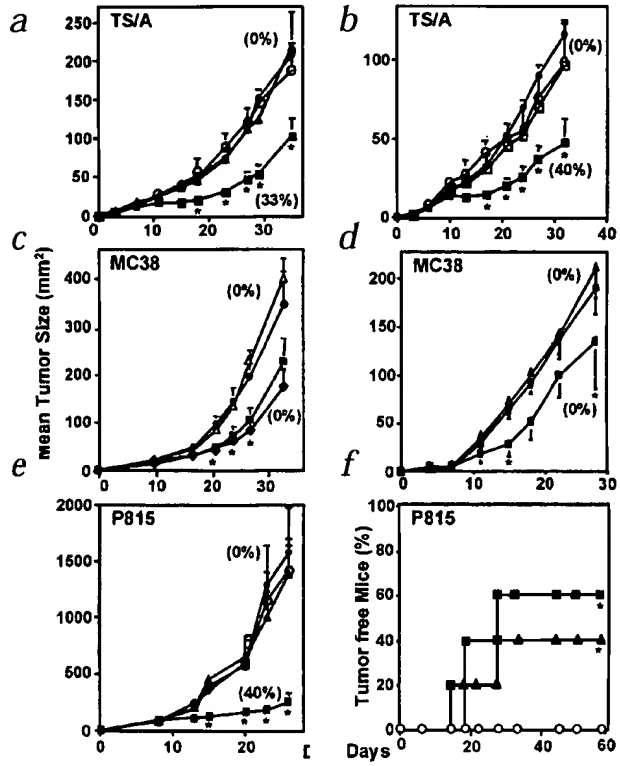

Fig. 3 Dendritic cells loaded with exosomes promote cross-protective antitumor effects. *a*, Day-3 TS/A-tumor-bearing BALB/c mice were immunized once with saline (●) or BM-DCS (BALB/c, H-2^d) loaded or not (○) with 15 μg of autologous TS/A (■) or allogeneic AK7 (▲) (H-2^b)-derived exosomes. *b*, Similar setting as *a* but immunization used BM-DC loaded or not (○) with 1μg (□) to 13 μg (■) of allogeneic MC38 (H-2^b)-derived exosomes. *c*, Day-3 MC38-tumor-bearing C57BL/6 mice were immunized with saline (●), BM-DC (H-2^b) loaded with 13 μg of autologous MC38-derived exosomes (◆), allogeneic TS/A (H-2^d)-derived exosomes (■) or with γ-irradiated TS/A-tumor cells (△). *d*, Similar setting as *c* but immunization used BM-DCs loaded with 13 μg of syngeneic AK7 (H-2^b)-derived exosomes (■) or with γ-irradiated AK7 tumor cells (△). *e*, Day-6–8 P815-tumor-bearing DBA/2 mice were immunized with BM-DCs (H-2^d, DBA/2) loaded or not (○) with 15 μg of autologous P815 (■) or syngeneic L1210 (H-2^d)-derived exosomes (▲). *f*, Similar setting as *e* but immunization used BM-DCS loaded or not (○) with 10–20 μg of autologous P815 tumor-derived exosomes(▲), or with semi-allogeneic Ba/F3p210 (H-2^d, BALB/c)-derived exosomes (■). Percentages of cured mice are indicated in parentheses. Each graph represents the data pooled from 3 independent experiments. 15 mice per individual group were used for statistical analyses. *, statistical significance at 95% confidence against negative controls (saline and/or dendritic-cells) using Fisher's exact method.

BALB/c) tumor-bearing mouse. Autologous tumor-derived exosome loaded BM-DCs promoted significant growth delay of the autologous tumors (Fig. 3*a*) and 33% cure rate at the end of the experiment in contrast with treatment with dendritic cells alone or saline (0% cures). AK7 mesothelioma allogeneic exosomes (H-2^b) or L1210 leukemia-cell-derived exosomes (H-2^d) did not cross-protect against the TS/A mammary adenocarcinoma (Figs. 3*a* and 4*a*, respectively) at similar dosage of exosomes, nor did they enable complete tumor regressions. In line with prophylaxis studies, allogeneic MC38 colon adenocarcinoma (H-2^b)-derived exosomes loaded onto syngeneic (H-2^d, BALB/c) BM-DCs mediated cross-protective antitumor effects against established TS/A with up to 40% tumor-free mice in contrast with dendritic cells alone or saline (0% tumor free animals; Fig. 3*b*). These antitumor effects required exosomes secreted from 20 million tumor cells (13 μg), pulsed onto 5 × 10^5 dendritic cells per mouse, whereas exosomes from 2 million tumor cells (1 μg) were insufficient in significantly reducing tumor growth (Fig. 3*b*). Antitumor cross-protection was reciprocal, as TS/A-derived exosomes could also mediate significant antitumor effects against the aggressive established MC38 tumor (Fig. 3*c*). Moreover, TS/A-derived exosomes loaded onto dendritic cells mediated antitumor effects on MC38 as efficiently as the autologous MC38-derived exosomes (Fig. 3*c*). In contrast, tumor-cell-de-

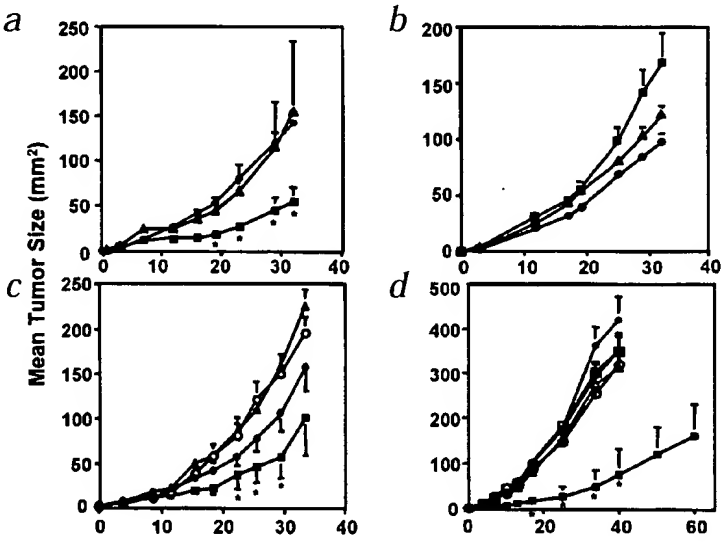

Fig. 4 Exosomes mediate CD4$^+$ and CD8$^+$ T-cell-dependent antitumor effects. *a* and *b*, As in Fig. 3*a*, day-3 TS/A (H-2^d)-tumor-bearing immunocompetent (*a*) or nude (*b*) BALB/c mice were immunized with 5 × 10^5 BM-DCs (H-2^d, BALB/c) loaded with 15 μg autologous TS/A (■) or semi-allogeneic L1210 (H-2^d, DBA/2)-derived exosomes (▲). *c*, Day-3 TS/A (H-2^d)-tumor-bearing mice were inoculated intradermally with MC38 (H-2^b)-derived exosome (10 μg)-pulsed dendritic cells with (△) or without (■) depletion of CD8$^+$ T cells (300 μg of YTS-169 4.2.1 mAb injected at day −1, +2, +8, +15 before/after tumor inoculation). Controls consisted of saline-treated mice depleted (○) or not (●) with YTS-169 4.2.1 mAb. *d*, Similar setting as in prophylaxis studies against TS/A in Fig. 2 using intradermal injections of MC38 exosomes (■) or saline (●) with injections of mouse serum. Depletion of CD8$^+$ T cells in mice receiving MC38 exosome (△) or saline (○) was performed using 300 μg of YTS-169 4.2.1 mAb. Depletion of CD4$^+$ T cells in mice receiving MC38 exosomes (◇) or saline (□) was performed using 300 μg of YTS-191 mAb. Depleting mAb or mouse serum was injected 3 days before the first immunization and twice a week until day 7 of tumor establishment. Each graph represents the data pooled from 3 independent experiments. 15 mice per individual group were used for statistical analyses. *, statistical significance at 95% confidence against controls (saline) using Fisher's exact method.

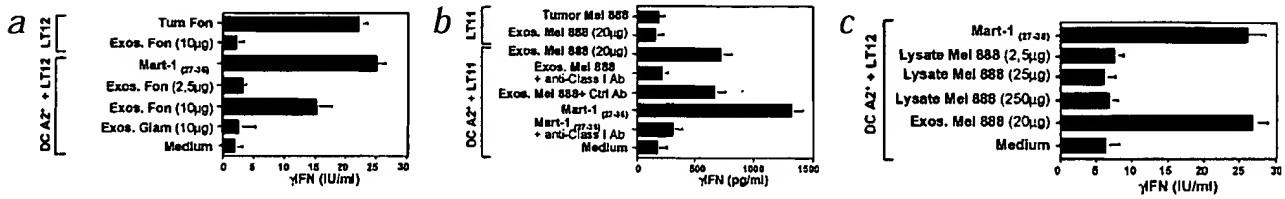

Fig. 5 Melanoma-derived exosomes transfer tumor antigens to MD-DCs. *a*, HLA-A2⁺ MD-DCs were incubated for 48 h with various dosages of Fon tumor-cell-derived exosomes or Mart-$1_{(27-35)}$ peptide (60 µM), or irrelevant Giam exosomes and used, in parallel to the autologous Fon tumor cell line (1×10^4/well) to trigger IFN-γ secretion from LT11 or LT12 clones. *b*, Similar setting as in *a* using exosomes derived from the HLA-A2⁻, Mart-1/MelanA⁺ Mel-888 line in the presence or absence of blocking mAb against MHC-I (W6.32) or isotype-matched control mAb (NKTa). *c*, HLA-A2⁺ MD-DCs were pulsed with Mel-888 exosomes or Mel-888 tumor lysates at indicated protein concentrations and co-incubated with the LT12 clone. Results are representative of 3 independent experiments, and indicated values represent the mean of duplicate wells (± s.e.m.).

rived lysates pulsed onto dendritic cells did not allow any cross-protections (data not shown).

Similarly, in a syngeneic setting, AK7 mesothelioma (H-2^b, C57BL/6)-derived exosomes exhibited significant antitumor efficacy against MC38 colon cancer (H-2^b, C57BL/6) when loaded onto syngeneic dendritic cells (H-2^b, C57BL/6) (Fig. 3*d*). P815 mastocytoma (H-2^d, DBA/2)-derived exosomes loaded onto syngeneic BM-DCs (H-2^d, DBA/2) were effective in reducing tumor growth kinetics (Fig. 3*e*) and eventually eradicating established P815 with up to 40 ± 20% long-term survival in the mice (Fig. 3*f*). Interestingly, whereas L1210 (H-2^d, DBA/2)-derived exosomes pulsed onto BM-DC did not impair P815 tumor growth, Ba/F3 leukemia (H-2^d, BALB/c)-derived exosomes pulsed onto BM-DC mediated complete cross-protection against day-7-established P815 with 60% long-term tumor-free survivors (Fig. 3*f*).

Altogether, tumor-derived exosomes allow efficient prophylactic and complete and reciprocal therapeutic intertumor cross-protections in rodent tumor transplantation models where irradiated tumor cells or necrotic cells (Figs. 2 and 3) are not immunogenic.

Exosomes induce T-cell–dependent antitumor immunity

In autologous settings (TS/A model), exosome-pulsed BM-DC mediate antitumor effects on established day-3—TS/A in immunocompetent animals (Fig. 4*a*), but not in Nu/Nu mice (Fig. 4*b*). In cross-protection settings, depletion of CD8⁺ T cells abrogated MC38-exosome-mediated antitumor effects on established TS/A (Fig. 4*c*). CD8⁺ T cells are also involved in *in vivo* cross-priming in prophylaxis studies using neutralizing monoclonal antibodies against CD8⁺ T cells (Fig. 4*d*). Moreover, depletion of CD4⁺ T cells also abrogated the MC38-exosome-mediated antitumor cross-protection against TS/A in prophylaxis studies (Fig. 4*d*). Altogether, both CD4⁺ and CD8⁺ T cells are required for *in vivo* cross-priming and intertumor cross-protection.

Human exosomes transfer tumor antigens to dendritic cells

The CD8⁺ T-cell–dependent cross-immunization and cross-protection indicate that exosomes transfer tumor antigens from tumor cells to APCs. We investigated cross-presentation of human tumor antigens contained in exosomes by MHC-I molecules of host human dendritic cells. We assessed the reactivity of V-β9/V-β2 LT11/LT12 HLA-A2-restricted and Mart-1/$MelanA_{27-35}$-specific clones[25] incubated with HLA-A2⁺ monocyte-derived dendritic cells (MD-DCs) loaded with melanoma exosomes bearing or not bearing HLA-A2 molecules. Most eukaryotic cells including melanoma cells possess MVB containing 60–90-nm vesicles enriched in CD63 [33,34];(data not shown). Such exosomes were collected from supernatants of two melanoma cell lines, Fon (HLA-A2⁺) and Mel-888 (HLA-A2⁻), already described to contain high amounts of cytosolic/melanosomal Mart-1/MelanA candidate tumor antigens. 10 µg of exosomes were secreted by $10–15 \times 10^6$ Fon or Mel-888 tumor cells over 48 hours. Fon but not Mel-888 cells trigger IFN-γ production from CTL (Fig. 5*a* and *b*). Interestingly, although they bear MHC-I molecules on their membranes, Fon-derived exosomes did not directly promote CTL activation *in vitro* (Fig. 5*a*). In contrast, when loaded onto HLA-A2⁺ MD-DCs, Fon-derived exosomes were almost as potent as micromolar ranges (60 µM) of synthetic Mart-1/$MelanA_{27-35}$ peptides in inducing IFN-γ production in the CTL clones (Fig. 5*a*). Importantly, a small quantity of melanoma-derived vesicles are secreted constitutively by melanoma cells *in vitro* (exosomes from 1×10^7 melanoma cells are used to pulse 1×10^4 dendritic cells to stimulate 1×10^4 CTLs). This result indicates that exosomes are capable of transferring MHC-I-peptide complexes and/or whole antigens to dendritic cells. To distinguish between both mechanisms, Mel-888-derived exosomes not bearing appropriate MHC-I-peptide complexes were loaded onto HLA-A2⁺ MD-DCs. Whereas Mel-888 did not stimulate LT11 clones, Mel-888-derived exosome-pulsed dendritic cells could efficiently promote CTL activation (IFN-γ production, Fig. 5*b*) in a MHC-I-dependent fashion, as assessed using neutralizing antibodies against MHC-I and not a control antibody, NKTa. In contrast, Giam (renal-cell carcinoma; RCC) derived exosomes (HLA-A2⁻, Mart-1/MelanA⁻) could not trigger LT11/LT12 following dendritic-cell loading. These data were confirmed using different CTL clones and at least four different sources of MD-DCs. Mel 888-derived lysates did not induce CTL reactivity in such settings, even at high dosages (Figs. 5*c* and 6*a*).

Exosomes could also elicit tumor-specific CTL reactivity in human tumor-infiltrating lymphocytes (TIL) in autologous settings of melanoma and RCC (data not shown). Whereas whole irradiated RCC cells failed to promote specific antitumor responses, RCC-tumor-derived exosomes elicited of tumor-specific recognition after 2 or 3 *in vitro* stimulations of TIL (data not shown).

Exosomes contain HSP70 and whole native tumor antigens

Anti-Mart-1-specific CTL activation strongly indicates that tumor-derived exosomes transfer Mart-1 tumor antigens to dendritic cells. Sucrose gradient separation of exosomal preparations and western blotting of the collected fractions revealed the presence of Mart-1/MelanA protein, mainly in fractions of 1.12 g/ml and 1.18 g/ml and corresponded to exosome density (Fig. 6*a*, right lane). In order to verify this, all fractions of the Mel-888 ex-

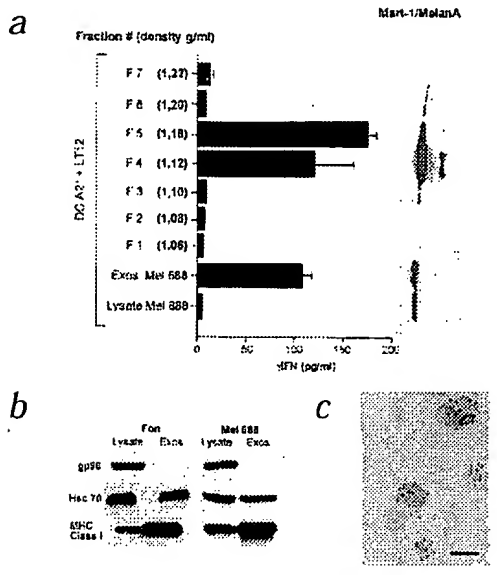

Fig. 6 Tumor-derived exosomes contain HSP70 and whole native tumor antigens. *a*, Sucrose density gradient analysis was performed on the exosomal pellets. Each individual fraction was run on SDS PAGE, immunoblotted with anti-Mart-1 Ab (A103) and tested in cross-presentation assays using similar methods as in Fig. 5. *b*, 48-h supernatant of 10–20 x 10^6 cells (Fon, left lanes, Mel-888 right lanes). 20 μg of exosomal proteins (Exos) or autologous tumor lysates were analyzed by western blotting using antibodies directed against MHC-I molecules, hsc70 and gp96. *c*, Whole-mount immunoelectronmicroscopy of exosomes derived from Mel-888-culture supernatant, labeled with anti-gp75 tyrosinase-related protein TRP mAb. Scale bars, 100 nm.

osomal pellets were tested in similar cross-presentation assays. Indeed, the bioactivity for CTL activation floated at 1.12–1.18 g/ml (Fig. 6*a*, left panel) and was resistant to proteinase-K digestion (data not shown). Western blot analysis of Fon- and Mel-888-derived exosomes revealed high levels of antigenic chaperones, that is, heat-shock proteins (HSP70-80; Fig. 6*b*) and the presence of MHC-I molecules (Fig. 6*b*). Constitutive rather than inducible HSPs were found accumulated in tumor-derived exosomes (as assessed after serum, glucose deprivation or heat shock of tumor cells) but ER-derived glycoprotein (gp)-96 was not detected. Interestingly, other differentiation tumor antigens such as tyrosinase-related proteins were found on melanoma exosomal membranes as assessed by immunoelectronmicroscopy studies using a specific antibody (Fig. 6*c*). Taken together, exosomes are vehicles of tumor antigens as native or misfolded proteins that are captured and processed by dendritic cells.

Discussion

Murine transplantation models do not support the hypothesis that shared tumor antigens are clinically relevant[8]. Here, we challenge this by describing a novel pathway of antigen transfer from tumor cells to dendritic cells. Using dendritic-cell-derived exosomes, we showed efficient protection and therapy of established murine tumors in syngeneic and allogeneic settings. Although shared tumor antigens were reported in mice, the dominant role of unique tumor antigens relevant in tumor rejection prevails, with little[35] if any[8] cross-protection among various tumor models. None of the previous cancer immunization strategies[11–18] achieved effective immunization across tumor types and MHC haplotypes. Here, irradiated tumor cells, apoptotic bodies or tumor lysates, were less effective than exosomes for protection against autologous tumors, and totally failed to promote any cross-protection. Tumor-derived exosomes loaded onto dendritic cells triggered T-cell-mediated antitumor immune responses leading to rejections of autologous tumors and strong intertumor cross-protections. Colon carcinoma (MC38)- and mammary carcinoma (TS/A)-derived exosomes, colon (MC38)- and mesothelioma (AK7)-derived exosomes, mastocytoma (P815)- and chronic myeloid leukemia (BAF)-derived exosomes were cross-protective, indicating that tumor-derived exosomes may contain shared tumor-rejection antigens. Cross-protections were reciprocal (TS/A and MC38; Figs. 2 and 3), and allogeneic exosomes were as efficient as autologous exosomes, excluding the possibility that MHC-I-peptide complexes present on tumor-cell-derived exosomes directly activated antitumor CTLs (Fig. 5). Importantly, not all tumor-derived exosomes are effective in therapy studies on irrelevant allogeneic tumors. However, our results indicate that exosomes concentrate a set of shared tumor-rejection antigens, which are efficiently taken up and cross-presented by MHC-I molecules on dendritic cells. Indeed, we show that Mart-1 melanoma antigens were cross-presented to specific T-cell clones after exosome uptake by human dendritic cells. Why then should exosomes be a relevant pathway for antigen cross-presentation?

First, exosomes display a discrete set of proteins. Exosomes were first described as vesicles released from maturing reticulocytes[36–38] and then also from hematopoietic APCs (refs. 19,30). In tumor cells, as indicated for EBV transformed-B lymphocytes[19] and dendritic cells[20], exosomes correspond to the internal vesicles of MVB. They originate from the inward invagination of a portion of the endosomal membrane and this process allows the engulfment of cytosolic proteins inside the 60–90-nm vesicles. Exosomes display a discrete set of proteins, different among tumors, and distinct from the total plasma-membrane or cytosolic proteins (Fig. 1*d*). As such vesicles are particularly enriched in HSP70 (Fig. 6*b*), part of the protective antitumor effects might be accounted for by such chaperones. The seminal work by Srivastava and colleagues[16] did not describe intertumor cross-protection *in vivo* using purified heat-shock proteins. As indicated in allograft models, it is possible that recognition of specific peptides presented by allogeneic MHC molecules with conserved TCR interaction sites might be as efficient as recognition of peptides presented by self-MHC molecules[39]. Hence, it remains to be determined whether MHC-I clustering on exosomal membranes might be an immunologically important alternative presentation pathway.

The second reason exosomes could be a pathway for cross-presentation is that they contain native tumor antigens. We show in melanoma cells that exosomes contain large amounts of differentiation antigens, that is, the cytosolic/melanosomal Mart-1/MelanA tumor antigens (Fig. 6*a*), as well as tyrosinase-related protein (Fig. 6*c*). The shared candidate tumor antigens relevant in our mouse models could not be found (that is, p53 and p210). In contrast, ovalbumin and green fluorescent protein were found in exosomes secreted by tumor cells transfected with the genes encoding such antigens (data not shown). The third reason is that exosomes overexpress targeting molecules and receptors potentially involved in sampling antigens to APCs. We observed effectiveness of exosomes in cases where tumor-cell derivatives from poorly immunogenic cancers (irradiated tumors

and lysates, Figs. 2, 3 and 5; apoptotic bodies, data not shown) did not trigger antitumor effects. Rather than reflecting quantitative differences in antigenic material transferred in each case, it is likely that exosomes express specific receptors or ligands for efficient uptake by APCs. Indeed, dendritic-cell-derived exosomes express molecules that might be candidates for a docking function[21,40]. The expression of such molecules on tumor-derived exosomes and their functional role require further investigation. Exosomes might have unique abilities to activate or stress APC to favor MHC-I cross-presentation pathways or to induce dendritic-cell maturation. As described for apoptotic debris, concomittant activation signals on dendritic cells[41] and/or CD4 T helper cells (M.L. Albert, pers. comm.) could be needed to elicit CTL cross-presentation. Nevertheless, using the D1 dendritic-cell line, we did not find that TS/A- or MC38-derived exosomes induce dendritic-cell maturation (data not shown).

The *in vivo* relevance of exosome release by tumor cells needs to be validated. Exosomes might be a tumor antigen-sampling compartment spread by tumor cells to modulate immunosurveillance. Tumor-derived exosomes are a novel source of tumor-rejection antigens and an original and efficient method of dendritic-cell loading that could be useful for the characterization of immunorelevant tumor antigens and for autologous or allogeneic cancer immunotherapy.

Methods

Dendritic-cell culture conditions. Human MD-DCs were obtained from the adherent fraction of peripheral blood mononuclear cells (PBMCs) as described[23]. PBMCs of HLA-A2$^+$ normal volunteers or melanoma patients were assayed by flow cytometry analysis with MA2.1 and PA2 antibody-containing ascites. Mouse BM-DCs were propagated as described[24] in culture medium supplemented with recombinant mouse interleukin 4 (rmIL-4) (10 ng/ml, R&D) and recombinant mouse granulocyte macrophage colony stimulating factor (rmGM-CSF) (1,000 IU/ml, R&D) and were pulsed at day 5 for 3 h at 37 °C with tumor-derived exosomes.

Tumor-cell culture and handling. Fon (HLA-A2$^+$, Mart-1/MelanA$^+$), originally propagated at the Gustave Roussy Institute[25] (IGR) and Mel-888 (HLA-A2$^-$, Mart-1/MelanA$^+$; provided by M. Maeurer) are two adherent human melanoma tumor cell lines propagated in RPMI 1640 supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 1 mM Na pyruvate (Gibco-BRL, France), and 20% and 10% decomplemented FCS (Seromed, France), respectively. Giam (HLA-A2$^-$, Mart-1$^-$) is a human renal-cell carcinoma line established at IGR from a primary adenocarcinoma[26]. The mouse cell line TS/A (H-2^d) is a moderately differentiated spontaneous mammary adenocarcinoma cell line syngeneic from BALB/c mice and provided by G. Forni. MC38 (H-2^b) is a highly agressive, spontaneous colon adenocarcinoma cell line, syngeneic from C57BL/6 mice, provided by M.T. Lotze. P815 (H-2^d) is a methylcholanthrene-induced mastocytoma cell line, syngeneic from DBA/2 mice. L1210 (H-2^d) is a leukemia cell line, syngeneic from DBA/2 mice. AK7 (H-2^b) is a highly agressive, MHC-I$^-$ (ref. 27), asbestos-induced mesothelioma cell line, syngeneic from C57BL/6, generated by A. Kane. Ba/F3 p210 (H-2^d) is a chronic myeloid leukemia (CML) cell line, syngeneic from BALB/c mice[28] resulting from the transfection of Ba/F3 with a retroviral construct recombinant for BCR/ABL. These cell lines were maintained mycoplasma-free in a culture medium (CM) of RPMI 1640 or DMEM (for AK7) supplemented with 10% endotoxin-free fetal calf serum (Gibco-BRL), 2 mM L-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin, essential amino acids and sodium pyruvate. Tumor models: 1×10^5 TS/A, 5×10^5 P815 or 2×10^5 MC38 were inoculated intradermally in the upper right abdominal flank of 6–8-wk-old female BALB/c, DBA/2 mice (Janvier Laboratories, Le Genest Saint Isle, France) and C57BL/6 mice (Iffa Credo Laboratories, Lyon, France) respectively. Monoclonal antibodies (mAb) against CD8α (clone YTS 169.4.2.1) and mAb against CD4 (clone YTS 191) provided by S. Cobbold[29] were used allowing 99% depletion. When irradiated, tumor cells received 5,000 rad.

Immunoelectron microscopy. Cells were fixed with 2% paraformaldehyde in Phosphate buffer pH 7.4. Cell pellets were embedded in 10% gelatin and infused in 2.3 M sucrose. Gelatin blocs were frozen in liquid nitrogen and ultrathin sections were collected as described elsewhere[30] (protein-A gold conjugates were purchased from J.W. Slot). Exosomes obtained after differential centrifugation of cell-culture supernatants were fixed in 4% paraformaldehyde and stored at 4 °C. Exosomes were loaded onto formvar-carbon-coated EM grids and immunogold-labeled and contrasted as described[19].

Exosome isolation. 48-h-supernatants of 80% confluent tumor cells were collected, and sequentially centrifuged (4 °C) at 300 *g* for 10 min, 800 *g* twice for 15 min, and then at 10,000 *g* for 30 min as described[19]. Exosomes were then pelleted at 100,000 *g* for 1 h, and washed once in PBS. The protein concentrations of exosomes were measured by Bradford assay (Biorad). Exosomes in amounts of 0.3–0.5 µg was usually obtained from $1–2 \times 10^6$ tumor cells in 48 h. Floatation of exosomes on a discontinuous sucrose gradient was performed at 4 °C as described[19] but using a SW41 rotor. Fractions of the gradient (700 µl each) were diluted in 3 ml PBS, centrifuged at 200,000 *g* for 2 h and each individual pellet was collected.

Western-blot analysis. Exosomal or cell lysate proteins were extracted as described[20] and analyzed by western blotting using anti-human MHC-I mAb (HC10, a gift from S. Ferrone), anti-constitutive heat shock protein 70 (anti-hsc70) (SPA 815, Stressgen, Victoria, Canada), anti-gp96 (SPA 850, Stressgen) or anti-Mart-1/MelanA (clone A103, Novocastra, New Castle upon Tyne, UK) at suppliers' recommended dilutions, followed by secondary horse radish peroxidase coupled antibodies and chemiluminescence revelation (Amersham, Meylan, France).

***In vitro* cross-presentation assays.** LT12 (V-β2) and LT11 (V-β9) are Mart-$1_{(27-35)}$-specific HLA-A2$^+$-restricted CD8$^+$ T-cell clones propagated as described[25]. Day 7 HLA-A2$^+$ MD-DCs were plated at 1×10^4 cells per well for 30 min with Mart-$1_{(27-35)}$ peptide (60 µM), Fon or Mel 888 or Giam exosomes. Blocking experiments were performed using mAbs agasint MHC-I, W6.32 IgG2a, or isotype-matched control mAb, NKTa (1/40 final dilution)[31]. 1×10^4 CTLs were added to 1×10^4 MD-DCs in a final volume of 200 µl in round-bottomed 96-well plates. 48-h supernatants were assessed in IFN-γ ELISA using Immunotech (Marseille, France) or OptEIA (Pharmingen, San Diego, Califonia) kits.

Acknowledgments

We thank F. André, A. Caignard and C. Bonnerot for helpful discussions; M.-F. Avril for providing us with melanoma patient-derived tumor material; A. Le Cesne for blood samples; S. Koscielny for statistical analysis; and the staff of the animal facility for animal care and handling. This work was supported by the Ligue Française de Lutte Contre le Cancer 'Axe Immunologie des Tumeurs', INSERM, CNRS, APCells SA and Inc., GEFLUC and Association pour la Recherche Contre Le Cancer ARC. J.W. was supported by Ligue Nationale de Lutte Contre le Cancer, A.L. by ARC.

RECEIVED 11 JULY; ACCEPTED 21 DECEMBER 2000

1. Boon, T. & Van der Bruggen, P. Human tumor antigens recognized by T lymphocytes. *J. Exp. Med.* **183**, 725–729 (1996).
2. Rosenberg, S.A. A new era for cancer immunotherapy based on the genes that encode cancer antigens. *Immunity* **10**, 281–287 (1999).
3. Gilboa, E. The makings of a tumor rejection antigen. *Immunity* **11**, 263–270 (1999).
4. Klein, G., Sjögren, H.O., Klein, E. & Hellström, K.E. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochtonous host. *Cancer Res.* **20**, 1561–1572 (1960).
5. Old, L.J., Boyse, E.A., Clarke, D.A. & A, C.E. Antigenic properties of chemically induced tumors. *Ann. NY Acad. Sci.* **101**, 80–106 (1962).
6. Old, L.J. Cancer immunology, the search for specificity. *Cancer Res.* **41**, 361–375 (1981).
7. Brändle, D. *et al.* The shared tumor-specific antigen encoded by mouse gene P1A is a target not only for CTL but also for tumor rejection. *Eur. J. Immunol.* **28**, 4010–4019 (1998).
8. Ramarathinam, L. *et al.* Multiple lineages of tumors express a common tumor antigen, P1A, but they are not cross-protected. *J. Immunol.* **155**, 5323–5329 (1995).
9. Zorn, E. & Hercend, T.A natural cytotoxic T cell response in a spontaneously re-

gressing human melanoma targets a neoantigen resulting from a somatic point mutation. *Eur. J. Immunol.* **29**, 592–601 (1999).
10. Imro, M.A. *et al.* Major histocompatibility complex class I restricted cytotoxic T cells specific for natural melanoma peptides recognize unidentified shared melanoma antigens. *Cancer Res.* **59**, 2287–2291 (1999).
11. Johnston, J.V. *et al.* B7-CD28 costimulation unveils the hierarchy of tumor epitopes recognized by the majority of HLA-A2-restricted CD8+ CTL. *J. Exp. Med.* **183**, 791–800 (1996).
12. Albert, M.L., Sauter, B. & Bhardwaj, N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86–89 (1998).
13. Russo, V. *et al.* DC acquire the MAGE3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response. *Proc. Natl. Acad. Sci.* **97**, 2185–2190 (2000).
14. Fields, R.C., Shimizu, K. & Mulé, J.J. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci.* **95**, 9482–9487 (1998).
15. Srivastava, P.K., Udono, H., Blachere, N.E. & Li, Z. Heat shock proteins transfer peptides during antigen processing and CTL priming. *Immunogenetics* **39**, 93–98 (1994).
16. Tamura, Y., Peng, P., Liu, K., Daou, M. & Srivastava, P.K. Immunotherapy of tumors with autologous tumor derived heat shock protein preparations. *Science* **278**, 117–120 (1997).
17. Boczkowski, D., Nair, S.K., Snyder, D. & Gilboa, E. Dendritic cells pulsed with RNA are potent antigen-presenting cells *in vitro* and *in vivo*. *J. Exp. Med.* **184**, 465–472 (1996).
18. Nair, S.K. *et al.* Induction of primary carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes *in vitro* using human dendritic cells transfected with RNA. *Nature Biotechnol.* **16**, 364–369 (1998).
19. Raposo, G. *et al.* B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **183**, 1161–1172 (1996).
20. Zitvogel, L. *et al.* Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nature Med.* **4**, 594–600 (1998).
21. Thery, C. *et al.* Molecular characterization of dendritic cell-derived exosomes: selective accumulation of the heat shock protein hsc73. *J. Cell Biol.* **147**, 599–610 (1999).
22. Escola, J.M. *et al.* Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B lymphocytes. *J. Biol. Chem.* **32**, 20121–20127 (1998).
23. Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* **179**, 1109–1118 (1994).
24. Zitvogel, L. *et al.* Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J. Exp. Med.* **183**, 87–97 (1996).
25. Dufour, E. *et al.* Diversity of the cytotoxic melanoma-specific immune response: some CTL clones recognize autologous fresh tumor cells and not tumor cell lines. *J. Immunol.* **158**, 3787–3795 (1997).
26. Angevin, E. *et al.* Human renal cell carcinoma xenografts in SCID mice: tumorigenicity correlates with a poor clinical prognosis. *Lab. Invest.* **79**, 879–888 (1999).
27. Fernandez, N.C. *et al.* Dendritic cells directly trigger NK cell functions: a cross-talk relevant in innate antitumor immune responses *in vivo*. *Nature Med.* **5**, 405–411 (1999).
28. Ahmed, M. *et al.* BCR-ABL and constitutively active erythropoietin receptor (cEpoR) activate distinct mechanisms for growth factor-independence and inhibition of apoptosis in Ba/F3 cell line. *Oncogene* **16**, 489–496 (1998).
29. Cobbold, S.P., Jayasuriya, A., Nash, A., Prospero, T.D. & Waldmann, H. Therapy with monoclonal antibodies by elimination of T-cell subsets *in vivo*. *Nature* **312**, 548–551 (1984).
30. Raposo, G. *et al.* Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol. Biol. Cell* **8**, 2631–2645 (1997).
31. Caignard, A. *In Situ* demonstration of renal-cell-carcinoma-specific T-cell clones. *Int. J. Cancer* **66**, 564–570 (1996).
32. Chen, L. *et al.* Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. *J. Exp. Med.* **179**, 523–532 (1994).
33. Wubbolts, R. *et al.* Direct vesicular transport of MHC class II molecules from lysosomal structures to the cell surface. *J. Cell Biol.* **135**, 611–622 (1996).
34. Grommé, M. *et al.* Recycling MHC class I molecules and endosomal peptide loading. *Proc. Natl. Acad. Sci.* **96**, 10326–10331 (1999).
35. Coggin, J.H.J. Cross-reacting tumor associated transplantation antigen on primary 3-methylcholanthrene-induced BALB/c sarcomas. *Mol. Biother.* **4**, 223–228 (1989).
36. Pan, B.T. & Johnstone, R.M. Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*: selective externalization of the receptor. *Cell* **33**, 967–977 (1983).
37. Johnstone, R.M., Matthew, A., Mason, A.B. & Teng, K. Exosome formation during maturation of mammalian and avian reticulocytes: evidence that exosome release is a major route for externalization of obsolete membrane proteins. *J. Cell Biol.* **147**, 27 (1991).
38. Rieu, S., Geminard, C., Rabesandratana, H., Sainte-Marie, J. & Vidal, M. Exosomes released during reticulocyte maturation bind to fibronectin via α4β1. *Eur. J. Biochem.* **267**, 583–590 (2000).
39. Stauss, H.J. Immunotherapy with CTLs restricted by nonself MHC. *Immunol. Today* **20**, 180–183 (1999).
40. Albert, M.L., Kim, J.I. & Birge, R.B. The alphavbeta5 integrin recruits the Crk/Dock180 molecular complex for phagocytosis of apoptotic cells. *Nature Cell Biol.* **2**, 899–905 (2000).
41. Sauter, B. *et al.* Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J. Exp. Med.* **191**, 423–433 (2000).